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NOTE

Characterization of a Phenotypically Unique Population of CD13$^+$ Dendritic Cells Resident in the Spleen

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Immature dendritic cells (DCs) resident in bovine spleens represent a distinct CD11a$^+$ CD11c$^+$ CD13$^+$ CD172$^+$ CD205$^+$ population compared to those circulating in peripheral blood or trafficking via afferent lymph. Upon cytokine-induced maturation, splenic DCs both efficiently present antigen in the stimulation of allogeneic lymphocyte proliferation and recall antigen-specific responses.

Dendritic cells (DCs) initiate adaptive immune responses by taking up an antigen and, following their maturation and migration to T-lymphocyte-rich zones, presenting the antigen to T cells in the context of the major histocompatibility complex (MHC) and costimulatory molecules (2, 31). Specific vaccine targeting of DCs has been shown to enhance priming and to convert nonimmunogenic tumor antigens into effective immunogens that induce protective immunity (3). Using DNA vaccine vectors expressing the Flt3 ligand and granulocyte-macrophage colony-stimulating factor (GM-CSF), along with a microbial antigen, we have shown that increased recruitment of DCs to the skin enhances the efficacy of DNA vaccines in cattle and that the CD4$^+$ T-lymphocyte responses are significantly amplified (23). However, there is strong evidence that priming, expansion, and maintenance of memory T lymphocytes and trafficking of these T cells upon challenge can be organ specific and are greatly influenced by the site of initial antigen presentation (6, 17, 21). Importantly, DC lineages differ among organs, and thus contemporary approaches that modify vaccine vectors to enhance the transfection and expression of vaccine antigens in organ-specific DCs may require different targeting strategies.

While DC lineages have been best studied in mice and humans, there is clear evidence for different lineages in cattle with functional differences in their abilities to stimulate CD4$^+$ and CD8$^+$ T cell responses (12, 28). However, these studies have not examined the spleen, the critical organ for priming and expansion of the immune response against blood-borne parasites (4, 8, 26, 27). Consistent with the critical role of the spleen in initiating protective immunity, splenectomy markedly delays the development of antigen-specific immune responses following infection with the blood parasites *Anaplasma* and *Babesia* spp., resulting in severe disease and, usually, death (16, 18). With a long-term goal of developing novel vaccines that will effectively induce immune responses that control these important hemoparasitic diseases of cattle, we are focused on improving our understanding of how immune responses are initiated and expanded in the spleen. The objective of the present study was to characterize splenic DCs and determine if they are phenotypically distinct from peripheral blood DCs and previously described bovine DC lineages obtained from afferent lymph (9, 12, 13).

Spleens were surgically removed from healthy male Holstein calves and, rinsed in phosphate-buffered saline (PBS) containing 20% (vol/vol) acid citrate dextrose (ACD) with 100 U penicillin and 100 μg streptomycin per ml. The spleen was mechanically disrupted using a tissue grinder, and cells were obtained by passing small fragments through a 100-μm-pore-size nylon cell strainer (BD Falcon). Spleen cells were centrifuged at 430 × *g* and resuspended in four volumes of Tris-buffered 0.87% ammonium chloride for 10 min. Remaining cells were washed in PBS-ACD, suspended in fetal bovine serum containing 10% dimethyl sulfoxide, and cryopreserved in liquid nitrogen. Peripheral blood mononuclear cells (PBMC) were isolated from the same calves and cryopreserved in liquid nitrogen using the same procedure as used for the spleen cells. B lymphocytes and monocytes were isolated from PBMC by positive selection using, respectively, the monoclonal antibodies (MAbs) BAQ44A and CAM66A (Table 1). Following 30 min of incubation at 4°C with the appropriate MAb, the cells were washed three times in PBS, incubated with goat anti-murine immunoglobulin M (IgM) microbeads (Milteny Biotec), and positively selected using a magnetic field. Macrophages were derived by culture of adherent PBMC in complete RPMI 1640 medium on 100-mm petri dishes (Becton Dickinson) at 37°C in 5% CO_2 for 7 days. Accutase (Innovative Cell Technologies) was used to collect adherent cells.

The strategy used to identify splenic DC populations was to

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TABLE 1. Monoclonal antibodies used for phenotypic analysis and cell sorting

Antibody	Isotype	Specificity	Source[a]
H34A	IgG2b	MHC class II	VMRD
TH22A	IgG2a	MHC class II	VMRD
BAQ44	IgM	B cell	VMRD
GB25A	IgG1	B cell	VMRD
CAM66A	IgM	CD14	VMRD
CAM36A	IgG1	CD14	VMRD
CACT31A	IgM	CD2	VMRD
BAQ95A	IgG1	CD2	VMRD
MM1A	IgG1	CD3	VMRD
CACT61A	IgM	γδ T-cell receptor δ chain	VMRD
CACTB81A	IgG1	γδ T-cell receptor γ chain	VMRD
GB21A	IgG2b	γδ T-cell receptor δ chain	VMRD
IL-11A	IgG2a	CD4	VMRD
CC30	IgG1	CD4	IAH
CACT80C	IgG1	CD8	VMRD
CC13	IgG1	CD1	Serotec
HUH73A	IgG1	CD11a	VMRD
MM12A	IgG1	CD11b	VMRD
BAQ153A	IgM	CD11c	VMRD
DH59B	IgG1	CD172a	VMRD
GC42A	IgG1	CD45RO	VMRD
ILA116	IgG3	CD45RO	IAH
CC81	IgG1	CD13	IAH
CC98	IgG2b	CD205	IAH
IL-A159	IgG1	CD80	IAH/EU
IL-A190	IgG1	CD86	IAH/EU
AKS1	IgG1	CD335 (NK cells [30])	NVS

[a] VMRD, Veterinary Medical Research and Development, Pullman, WA; IAH, Institute for Animal Health, Compton, England, United Kingdom (provided by Chris Howard); EU, Edinburgh University, Edinburgh, Scotland, United Kingdom (provided by N. MacHugh); NVS, Norwegian School of Veterinary Science, Oslo, Norway (provided by A. K. Storset).

select cells expressing MHC class II molecules and then to remove those other cell subsets that also express MHC class II molecules: monocytes, macrophages, B lymphocytes, αβ T lymphocytes, γδ T lymphocytes, and NK cells (30). The remaining MHC class II molecule-expressing population, putative DCs, was then analyzed morphologically for characteristic dendrite formation, phenotypically for cell surface markers expressed by other bovine DC lineages, and functionally for the ability to present antigen and stimulate lymphocyte responses. This approach was necessary as, in the absence of prior studies of bovine splenic DCs, there are no lineage-specific markers; this approach was also necessary to avoid the bias that all bovine DCs, regardless of lineage, share a common cell surface marker. For sorting, spleen cells and PBMC were incubated with two sets of isotype-specific primary MAbs: (i) IgM MAbs specific for bovine CD2 (expressed on αβ T lymphocytes and NK cells), B lymphocytes, γδ T lymphocytes (δ-chain specific), and CD14 and (ii) IgG2a MAb specific for bovine MHC class II (HLA-DR orthologue) (Table 1). After incubation for 30 min on ice, cells were washed three times in cold PBS by centrifugation at 430 × *g* and then incubated with the secondary antibodies, phycoerythrin (PE)-conjugated goat anti-murine IgM and fluorescein isothiocyanate-conjugated goat anti-murine IgG2a, for 15 min on ice. Cells were washed twice in cold PBS, and then putative DCs that were MHC class II positive but negative for CD2, CD14, B-lymphocyte, and γδ T-lymphocyte markers were gated and sorted using a Vantage fluorescence-activated cell sorter (Becton Dickinson) (Fig. 1).

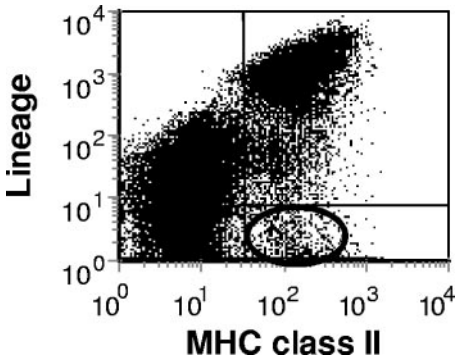

FIG. 1. Isolation of bovine splenic DCs. MHC class II^+ cells that were negative for the expression of CD2, CD14, and B- and γδ T-lymphocyte markers (Lineage) were gated (circled population) for isolation by sorting.

Sorted putative splenic DCs were then cultured in complete RPMI 1640 medium supplemented with 200 ng/ml of recombinant bovine interleukin 4 (IL-4) (10, 24), 100 ng/ml recombinant bovine GM-CSF (23, 24), 100 ng/ml recombinant bovine Flt3 ligand (22, 24), and 10 μg/ml recombinant bovine CD40 ligand (rbCD40L). To obtain bovine CD40L, a DNA construct encoding the extracellular domain of bovine CD40L (CD40L-ED) linked in-frame with the sequence encoding the CD5 secretory signal sequence (7) was generated in the expression vector VR-1055 (Vical). A bovine CD40L-ED-specific forward primer (5′ ATACTGCAGATGGTGTATCTTC ACAGACGATTG 3′) and a bovine CD40L reverse primer (5′ ATA*GGATCC*TCA**CTTATCGTCATCGTCCTTGTAGTC**CC CTGGACCAGGTCCGAGTTTGAGTAAGCCAAATGA 3′) were used to PCR amplify the CD40L-ED open reading frame from cDNA generated from bovine T lymphocytes stimulated with ionomycin and phorbol myristic acetate as previously described (11). The reverse primer was extended to include complementary sequence (in bold) of the codons encoding the FLAG tag (amino acid sequence, DYKDDDDK) (20) and also introduced a BamHI restriction site (in italics) at

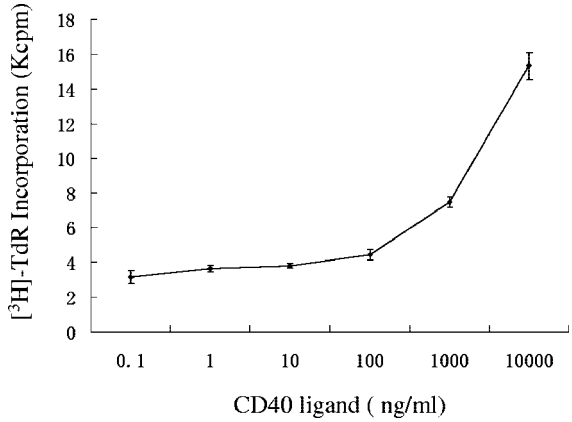

FIG. 2. Bioassay of recombinant bovine CD40L. The biological activity of rbCD40L was tested by its ability to stimulate B-lymphocyte proliferation in the presence of 200 ng/ml recombinant bovine IL-4 (10, 24). Means ± standard deviations of results from triplicate wells are shown. TdR, thymidine.

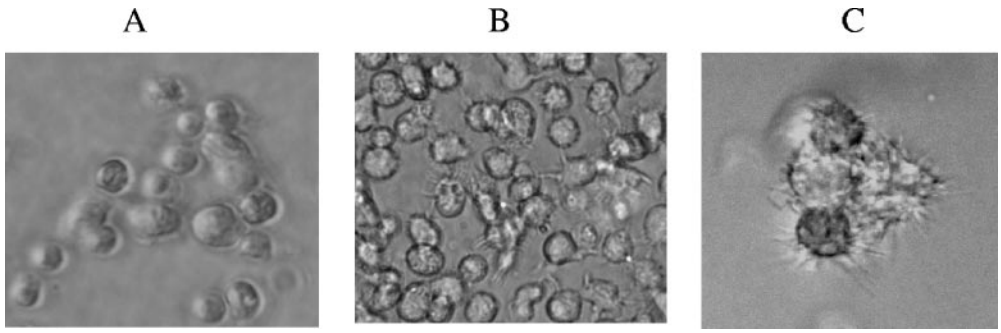

FIG. 3. Morphology of bovine splenic DCs. Immature DCs immediately after sorting (A) and mature DCs after 72 h of culture at magnifications of ×300 (B) and ×600 (C) are shown.

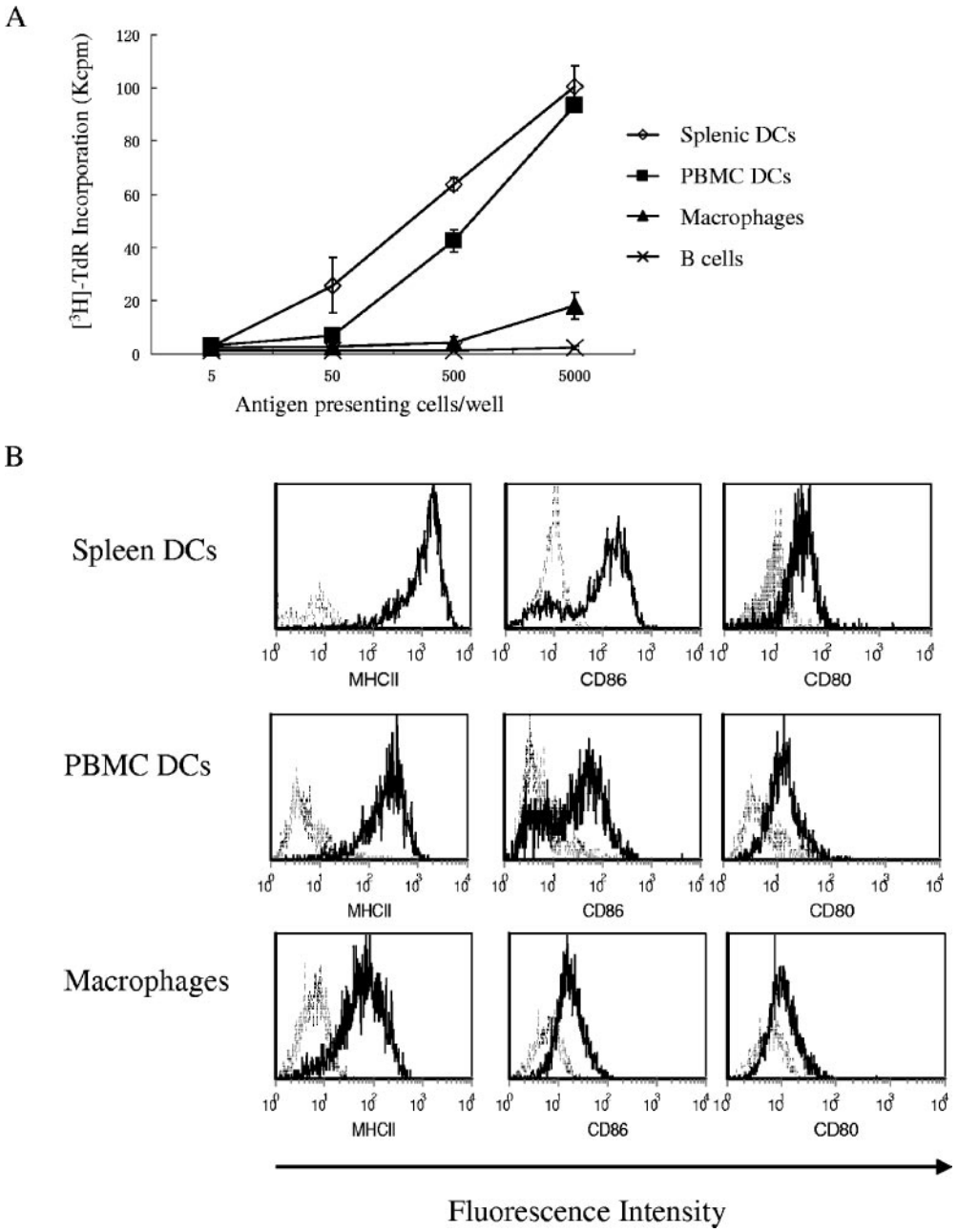

FIG. 4. Comparison of the abilities of different antigen-presenting cells to stimulate an allogeneic lymphocyte response and correlation with activation phenotype. (A) Stimulation of allogeneic lymphocyte proliferation by increasing numbers of mature splenic or peripheral blood DCs, macrophages, B lymphocytes, or monocytes. Means ± standard deviations of results from triplicate wells are shown. The response induced by monocytes was identical to that of B lymphocytes and thus is not shown. (B) MHC class II, CD80, and CD86 surface molecule expression on mature splenic and peripheral blood DCs and macrophages (black profile). The background of secondary antibody binding is indicated by the gray profile. TdR, thymidine.

the 3′ end of the PCR product, designated cd40l$_{ed}$flag. The CD5 secretory signal sequence was added at the 5′ end of cd40l$_{ed}$flag by PCR using two overlapping primers (5′ ACCT TGTACCTGCTGGGGATGCTGGTCGCTTCCTGCCTCG GACTGCAGATGGTGTATCTTCACAGACG 3′ and 5′ AT **AGATATC**ACCATGCCCATGGGGTCTCTGCAACCGCT GGCCACCTTGTACCTGCTGGGGATGCTG 3′), and the second primer introduced an EcoRV restriction site (in bold) at the 5′ end of the PCR product. The resultant construct, designated cd5cd40l$_{ed}$flag, was EcoRV-BamHI digested and subcloned into the VR-1055 eukaryotic expression vector to generate a construct designated CD40L-ED. rbCD40L was expressed as FLAG-tagged protein in 293 Free-Style cells (Invitrogen) and affinity purified using Anti-FLAG M2-agarose gel (Sigma) as previously described (24). Purified protein was then tested for biological activity with B lymphocytes positively selected with magnetic beads from PBMC using a modified protocol of a previous study (29). Briefly, B lymphocytes (2×10^5 cells/well) were incubated in triplicate with 200 ng/ml recombinant bovine IL-4 (10, 24) either alone or in combination with rbCD40L in a concentration range between 0.1 ng to 10 μg per ml. Cells were incubated for 72 h at 37°C with 5% CO_2, and proliferation was measured by radiolabeling with 0.25 μCi of [^{3}H]thymidine over the last 18 h of culture. Cells were collected using an automated cell harvester (Tomtec), and incorporated [^{3}H]thymidine was counted with a liquid scintillation counter. The stimulation of significant dose-dependent proliferation of B lymphocytes (1, 19) demonstrated that the recombinant cytokine was biologically active (Fig. 2).

Microscopic examination of the putative splenic DCs immediately after sorting revealed a rounded appearance (Fig. 3A). Small dendrites appeared following overnight culture, and numerous long cytoplasmic veils and aggregates, typical of fully mature DCs, developed within 72 h (Fig. 3B and C). To examine the ability of these mature DCs to present antigen and stimulate T cells, two assays were performed. The first was an allogeneic T-cell stimulation in which sorted splenic DCs, DCs sorted from PBMC, B lymphocytes, macrophages, and monocytes (obtained from calves with the MHC class II DRB3 **0101/*1201* alleles) were irradiated and individually cultured with 1×10^5 lymphocytes (obtained from a calf with MHC class II DRB3 **4501/*3001* alleles) in round-bottom 96-well plates in 100 μl of complete RPMI 1640 medium for 96 h at 37°C in 5% CO_2. Proliferation was measured by adding 0.25 μCi [^{3}H]thymidine for the final 18 h of cultivation, followed by cell harvesting and liquid scintillation counting as described above. DCs sorted from the spleen and from peripheral blood induced significantly higher ($P < 0.05$) proliferative responses of allogeneic lymphocytes than did purified macrophages, monocytes, or B lymphocytes (Fig. 4A). The magnitude of lymphocyte proliferation was proportional to the number of DCs added (Fig. 4A) and the comparative effectiveness of the DCs as antigen-presenting cells associated with the surface expression of MHC class II, CD80, and CD86 molecules (Fig. 4B).

The second functional assay measured the ability of DCs to stimulate antigen-specific recall responses. A short-term T-lymphocyte cell line was established from a calf (MHC class II DRB3 **1201/*1201*) immunized with *Anaplasma marginale*

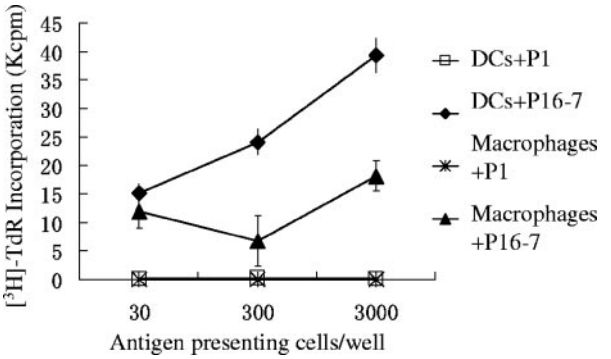

FIG. 5. Comparison of the abilities of mature splenic DCs and macrophages to induce an antigen-dependent T-lymphocyte recall response. Specific MSP2 peptide antigen (P16-7) or a control peptide (P1) was added to either splenic DCs or macrophages for presentation to an MSP2-specific T-cell line (25). Means ± standard deviations of results from triplicate wells are shown.

MSP2 protein (5). Briefly, CD8- and γδ T-cell-depleted PBMC were stimulated with MSP2 for 1 week, followed by a 1-week rest as previously described (23). The specific MSP2 peptide P16-7, previously shown to be presented by the MHC class II DRB3 **1201* allele (25), or a negative control peptide, P1, were added to autologous splenic DCs or macrophages as antigen-presenting cells and cultured with 3×10^4 cells of the T-lymphocyte cell line. After 72 h, cells were pulsed with [^{3}H]thymidine and harvested as described above. Both splenic DCs and macrophages presented antigen and induced antigen-specific CD4$^+$ T-cell recall responses, with significantly higher responses ($P < 0.05$) stimulated by DCs when either 300 or 3,000 antigen-presenting cells were used (Fig. 5).

To identify the surface phenotype of these splenic DCs, monoclonal antibodies recognizing a series of both activation markers and surface markers previously identified on bovine afferent lymph DCs were used in multicolor flow cytometry. DCs (MHCII$^+$ CD2$^-$ B cell$^-$ γδ$^-$ CD14$^-$) derived from either peripheral blood or spleen were incubated with MAbs specific for each of the following cell surface markers: CD1, CD11a, CD11b, CD11c, CD13, CD45RO, CD80, CD86, CD172a, and CD205 (Table 1). After incubation for 30 min on ice, cells were washed three times in complete RPMI 1640 medium containing 0.02% sodium azide by centrifugation at $300 \times g$. Fluorescein isothiocyanate-, PE-, allophycocyanin-, or PE-Cy5-conjugated isotype-specific goat anti-mouse antibodies were used as secondary antibodies (Caltag Laboratories and Southern Biotech). After incubation for 15 min on ice, labeled cells were washed twice in cold PBS and fixed in 1% formaldehyde. A minimum of 80,000 labeled cells were analyzed by flow cytometry using a FACSort (Becton Dickinson). Splenic and peripheral blood DCs were clearly distinct from the B lymphocytes, monocytes, and macrophages, being negative for these lineage-specific markers (data not shown). The splenic DCs were phenotypically distinct from both the peripheral blood DCs and the two previously described types of afferent lymph DCs (Table 2). Between DCs derived from peripheral blood versus spleen, the major surface phenotypic difference was the high level of CD13 (14) expression on the splenic DCs (Fig. 6). The expression of this marker, which is a type II transmembrane

TABLE 2. Comparison of bovine DC surface phenotypes

Surface molecule	Surface phenotype[a]			
	Spleen DCs[b]	Peripheral blood DCs[b]	Afferent lymph DC subset 1[c]	Afferent lymph DC subset 2[c]
MHC class II	++	++	++	++
CD1b	−	−	+	++
CD2	−	−	−	−
CD4	−	−	−	−
CD8	−	−	−	−
CD11a	++	++	+	−
CD11b	±	+	−	−
CD11c	++	+	±	+
CD13	++	−	+	−
CD21	−	−	−	− or +
CD45RO	−	−	+	+
CD80	±	±	++	++
CD86	−	−	++	++
CD172a	+	+	−	+
CD205	++	+	++	++

[a] Intensities of monoclonal antibody binding are indicated as − (negative), ± (weak), + (positive), or ++ (strongly positive), according to the conventions of Howard and Hope (13). In this study, these symbols are defined as follows: ++, ≥60% of cells were positive; +, 15 to 59% of cells were positive; ±, <15% were positive; and −, cells were negative.

[b] Splenic and peripheral blood DCs were analyzed as uncultured cells using three-color flow cytometry.

[c] Data are from references 12 and 13. Uncultured cells were examined.

protein, was not linked to differential activation status, as both sets of DCs revealed very low or no expression of CD80 or CD86 (Table 2). Splenic DCs also displayed expression levels of CD11b, CD11c, and CD205 different from those of peripheral blood DCs (Fig. 6). CD205, a type I cell surface glycoprotein, is expressed on different DC lineages in mice (15) and has been used as a lineage marker to isolate DCs from the large-sized bovine afferent lymph veiled cells (9, 12, 13). Examination of total gated $CD205^+$ cells in the bovine spleen included both B ($CD21^+$) and T lymphocytes ($CD3^+$) (data not shown). Thus, although the splenic DCs uniformly expressed CD205, CD205 cannot be used as a specific DC marker or as a specific targeting molecule for vaccine delivery. Splenic DCs were also distinct from the two well-described subsets of afferent lymph DCs (12, 13) based on multiple discriminatory surface markers (Table 2). Splenic DCs did not express CD45RO or CD1, which are expressed on both subsets of afferent lymph DCs. Uncultured splenic DCs also exhibited an immature phenotype, as indicated by the low level of CD80/86 expression, while the higher level of expression on the afferent lymph DCs is indicative of a more mature phenotype.

Maturation of splenic DCs using IL-4, the Flt3 ligand, GM-CSF, and CD40L induced strong expression of both CD80 and CD86 (Fig. 4B) concomitantly with a complete loss of CD13 (data not shown). This mature phenotype was linked to the capacity of the DCs to present antigen effectively (Fig. 4A and B). Comparison with identically cultured peripheral blood DCs revealed differences in expression of all three CD11 molecules: mature splenic DCs had higher levels of CD11a and CD11c expression but lower levels of CD11b than mature peripheral blood DCs. Thus, the two populations of DCs retain distinct cell surface phenotypes following maturation.

In the present study we have identified a distinct $CD13^+$ DC population in bovine spleens that is phenotypically unique compared to other peripheral blood, lymph node, and afferent lymph DCs and confirmed its effectiveness in antigen presentation in both the allogeneic T-cell stimulation reaction and the induction of T-cell recall responses. Unlike the two subsets

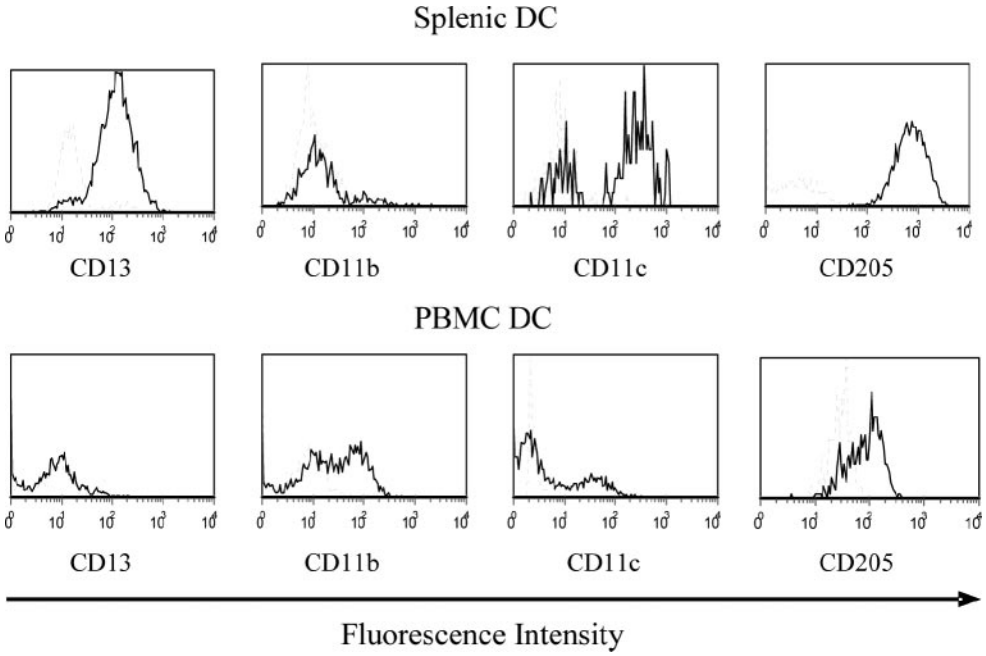

FIG. 6. Splenic and peripheral blood DCs display distinct cell surface phenotypes. Uncultured DCs were analyzed by flow cytometry. Levels of expression of CD13, CD11b, CD11c, and CD205 are indicated by the solid line. The background of secondary antibody binding is indicated by the light gray dotted lines.

of bovine afferent lymph DCs, which differ in surface phenotype, including in the expression of CD11a, CD13, and CD172a, there was no definitive evidence of multiple splenic DC subsets when the cells were examined either prior to or following activation. However, minor subsets not detected in normal spleens from healthy animals may well be identified if there is specific expansion or activation by delivery of antigens, including the blood-borne pathogens *Anaplasma* and *Babesia*, to the spleen. Determining how splenic DCs are activated and migrate following the initial encounter with the pathogen and how priming in the spleen affects T-cell trafficking represent the next challenges for better understanding immunity against these important pathogens.

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